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Award Number: W81XWH-11-1-0228

TITLE: Tumor Microenvironment and Progression to Invasion after a Diagnosis of Ductal Carcinoma In situ

PRINCIPAL INVESTIGATOR: Patricia J. Keely, PhD
Co-PI: Amy Trentham-Dietz, PhD

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, WI 53715-1218

REPORT DATE: March 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
31-03-2012	Annual	01 Mar 2011 – 28 Feb 2012

4. TITLE AND SUBTITLE

Tumor Microenvironmnet and Progression to Invasion after a Diagnosis of Ductal Carcinoma In Situ

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0228

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Amy Trentham-Dietz and Patricia Keely

E-Mail: trentham@wisc.edu and pjkeely@wisc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Wisconsin
Madison, WI 53715-1218

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Ductal carcinoma in situ (DCIS) makes up 18% of all new breast cancer diagnoses, and is considered a precursor to invasive breast cancer even though the majority of cases—almost 70%—may never progress to invasive disease. Markers that identify which patients are most likely to experience progression are critically needed so that fewer patients are over-treated. This study is evaluating two novel tumor markers that may indicate greater risk of tumor progression based on recent work that suggests that stromal syndecan-1 expression induces an extracellular matrix with an aligned collagen fiber architecture, and that this collagen alignment in turn facilitates malignant cell invasion. We are using archived tumor tissue from 267 cases of DCIS of the breast to evaluate syndecan-1 expression and collagen alignment. These DCIS cases, diagnosed between 1995 and 1999, have been followed for breast cancer outcomes; to-date, 12.5% of cases have experienced a second breast cancer diagnosis. Analysis of syndecan-1 expression and collagen alignment patterns is on-going. Data analysis will ensue upon completion of tumor markers. No scientific knowledge has yet been produced.

15. SUBJECT TERMS
Ductal carcinoma in situ, breast cancer, tumor microenvironment, collagen alignment, syndecan-1, disease-free survival, second harmonic generation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	8	

TABLE OF CONTENTS

INTRODUCTION

Ductal carcinoma in situ (DCIS) makes up 18% of all new breast cancer diagnoses, and is considered a precursor to invasive breast cancer. It is estimated that almost 70% of DCIS cases may never progress to invasive disease. However, since the transition from DCIS to invasive breast cancer is a critical progression step associated with a substantial drop in survival, patients are uniformly treated with aggressive therapy, and thus many are being over-treated. Unfortunately, relatively little is known about the factors that govern this progression, and so markers that isolate patients likely to progress have not been identified. An emerging approach in tumor biology focuses on important changes in the stromal tissue surrounding malignant cells during tumor progression. The recent work of Drs. Patricia Keely and Andreas Friedl with invasive breast carcinoma suggests that stromal syndecan-1 expression induces an extracellular matrix with an aligned collagen fiber architecture, and that this collagen alignment in turn facilitates malignant cell invasion. These changes have not been investigated in DCIS. We hypothesize that re-alignment of the extracellular matrix, triggered by syndecan-1 induction in stromal fibroblasts, plays a major role in the progression from DCIS to invasive breast cancer, and thus can be used as a marker to predict outcome. Our objective is to evaluate this hypothesis using archived tumor samples and follow-up data from Dr. Amy Trentham-Dietz's cohort study of 267 DCIS cases with available tumor samples who were recruited upon their diagnosis between 1995 and 1999. Collagen patterns and stromal expression of syndecan-1 will be evaluated from archived unstained tumor slides using state-of-the-art methods by Drs. Keely and Friedl, respectively.

BODY

The approved Statement of Work for this grant includes:

Task 1. **Obtain and maintain regulatory approval, Months 1-24:**

a. Obtain initial IRB/Human Subjects approvals, Months 1-6.

<u>Progress report</u>: Initial IRB/human subjects approval was obtained in August 2011 (month 6) from both the University of Wisconsin (UW) Health Sciences IRB and the DOD Human Research Protection Office (HRPO). IRB approval will expire at the UW on 16 May 2012 (month 15).

b. Obtain continuing review annual approval from IRB, Month 12.

<u>Progress report</u>: The annual progress report was submitted to the UW Health Sciences IRB on 20 March 2012 (month 13). A copy of the continuing review approval notification by the University of Wisconsin Madison Minimal Risk IRB (Health Sciences) will be submitted to the HRPO as soon as possible after receipt of approval.

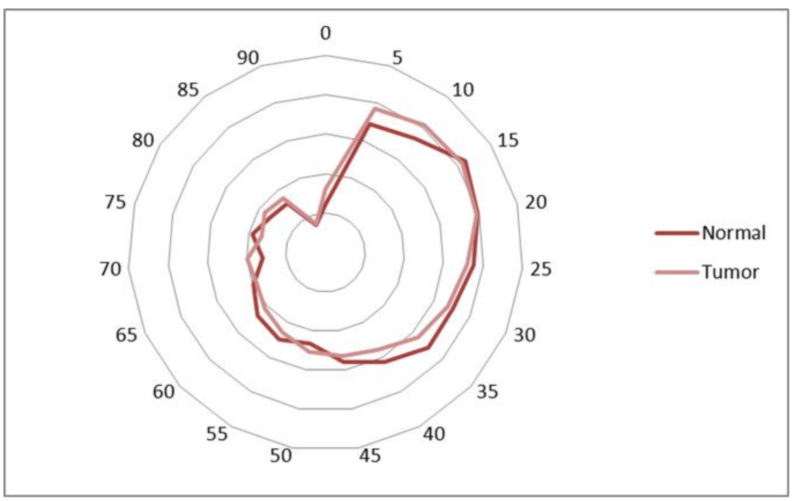
Task 2**. Evaluate tumor microenvironment in 267 DCIS samples, Months 7-10;**

a. Evaluate collagen alignment patterns, Months 5-10.

<u>Progress report</u>: Evaluation of the collagen alignment patterns has begun. Procedures for assessment of collagen alignment were first conducted on tissue slides for 16 cases. Collagen fibers were imaged using second harmonic generation (SHG) microscopy, which is a non-linear optical imaging form of microscopy. The technique takes advantage of the unique non-centrosymmetric structure of collagen in combination with the multiphoton

absorbance of laser light by the peptide bonds of collagen to act as a frequency doubler. The net effect is that the emitted light is of exactly one-half the wavelength of the incident light upon interaction with collagen. In this way, an image of the collagen extracellular matrix (specifically) is acquired. These images were then transformed in the frequency space into curvelets, which are essentially vector representations of individual collagen fibers. A boundary between the tumor and stroma was drawn in the image by the user and the "curvelets" software program then measured the angle at which each curvelet crossed the border. These individual measurements were compiled to create a histogram of the angles at which collagen fibers are oriented with respect to the tumor boundary. Since there are many fibers at any given lesion, this automated analysis is highly useful. The multiphoton microscope and curvelet analysis program used were both custom created through established collaborations here at the University of Wisconsin.

Collagen alignment was evaluated in 1-4 tumor lesions and compared to adjacent normal cells for the 16 cases. For these 16 cases, we compared the angles at which the collagen fibers were configured relative to the tumor boundary. Statistical analysis designed for compositional data demonstrates that the distribution of collagen fiber angles for the tumor lesions was significantly different than the pattern of collagen fiber angles for the adjacent normal cells (P=0.0054). As shown in the figure below, in both normal and tumor, many of the fibers were aligned at 5 to 45-degree angles; relatively few of the lesions were aligned at 70 to 85-degree angles, and relatively few fibers were aligned at zero or 90-degree angles. Relative to normal, tumor lesions had relative increases at angles 0-10, 50, 70, 80-90. Of these, the increases for 0 & 70 are most prominent. The other angles show relative drops (fiber alignments were lower for tumor relative to normal for 15 to 45-degree angles). Of these, the drops for 35-40, 60 & 75 degree angles are most prominent.



Now that procedures for evaluating collagen alignment have been established, fiber alignment patterns for the remaining DCIS cases will commence. It is expected that evaluation of fiber alignment patterns of all cases will be completed by July 2012.

b. Evaluate syndecan-1 expression, Months 5-10.

Progress report: Following initial optimization, immunohistochemical labeling for syndecan-1 has been completed for all 267 cases. Syndecan-1 expression by fibroblasts located in the periductal stroma will be assessed either semi-quantitatively by manual scoring or quantitatively using the Nuance image analysis system. We expect completion of the analysis by the end of June 2012 (month 16).

Task 3. **Determine outcome status among 267 DCIS cohort subjects, Months 7-8.**

a. Clean study cohort dataset to determine 2nd breast cancer events, Months 7-8.

Progress report: Follow-up of cohort subjects is on-going. To-date, we have ascertained 32 second breast cancer diagnoses (12.5%) among 255 DCIS cases. Some of the cases (N=12) may be excluded from analyses since review of that pathology reports corresponding to the initial diagnoses suggest that the tumors were not DCIS but either benign or invasive breast cancer. These 12 cases will be centrally reviewed by our co-investigator (A. Friedl) to determine final eligibility.

b. Categorize 2nd breast cancer events by invasive/in situ stage, ipsilateral/contralateral location, and estrogen receptor status, Months 7-8.

Progress report: Among the 32 second diagnoses among the 255 DCIS cases, 34% were invasive breast cancer, 53% were *in situ* breast cancer, and 13% (N=4) have unknown extent of disease (See table). A slight majority of second diagnoses were ipsilateral (53%). Estrogen receptor (ER) status is unknown for most of the second diagnoses (69%); 90% of the second diagnoses with known ER status were ER-positive.

Summary of DCIS cases (median length of follow-up = 11.8 years)

	N	%
Total	255	
Second diagnoses	32	12.5%
Extent of disease		
Invasive	11	34%
In situ	17	53%
Unknown	4	13%
Laterality		
Ipsilateral	17	53%
Contralateral	13	40%
Both	2	6%
Unknown	1	3%
Estrogen receptor status		
Positive	9	28%
Negative	1	3%
Unknown	22	69%

Task 4. **Statistical analyses, Months 11-17:**

a. Link the tumor microenvironment data from Task 2 to the cohort data of Task 3, Month 11.

b. Characterize the collagen alignment patterns and syndecan-1 expression levels in the 267 DCIS samples, Months 11-12.

c. Evaluate the association between the tumor microenvironment data and tumor/patient characteristics, Months 13-15.

d. Determine the relation between the tumor microenvironment data and disease-free survival, Months 16-17.

Progress report: Statistical analyses utilizing the collagen alignment and syndecan-1 expression levels will commence once data collection is complete. It is expected that Task 4 will be completed by month 20 (October 2012).

To prepare for these analyses, we have characterized disease-free survival in the WISC Cohort from which the DCIS cases with tumor tissue samples were drawn. There are 1,959 DCIS cases in the WISC Cohort. During a median of 6.3 years of follow-up, 133 second breast cancer events were recorded among these cases. Overall disease-free survival was about 95% at 5 years. Disease-free survival varied according to treatment received, ranging from 86.5% among cases receiving no further treatment after biopsy to 100% among women receiving a bilateral mastectomy. Women receiving breast conservation surgery followed by radiation had similar disease-free survival (95.4%) compared to women undergoing ipsilateral mastectomy (94.7%). Tamoxifen use reduced the risk of a second event by about 20% among all treatment groups, with the exception of those undergoing bilateral mastectomy. We have also examined tumor and patient characteristics in relation to DCIS disease-free survival in the WISC Cohort. DCIS cases detected symptomatically were more likely to have a recurrence than cases detected by screening mammography (HR=1.6; 95% CI 0.9-3.0). Tumor size, grade, and histologic subtype were not strongly associated with disease-free survival. Similarly, little variation in disease-free survival was observed by age, family history of breast cancer, body mass index, parity, postmenopausal hormone use, and education. These findings will be updated following the most recent round of data collection for the parent study. These results provide information as to the inclusion of potentially confounding factors in the analyses of Task 4.d. Our findings suggest that treatment received and mode of detection are important variables for inclusion in multivariable models of tumor microenvironment in relation to disease-free survival. The overall disease-free survival rates in the parent cohort will also be informative in comparing the disease-free survival rates observed among the DCIS cases with tumor tissue samples (i.e., to guide the generalizability and interpretation of the results).

***Task 5*. Communication of results, Months 12-24:**

a. **Submit annual progress report to the DOD, Month 12**
b. **Prepare manuscripts describing the results found in Task 4, Months 18-24.**
c. **Present the study results at the DOD Era of Hope meeting and other national conferences, Months 12-24.**
d. **Deliver final report to the DOD, Month 24.**

Progress report: Communication of results will commence once data are collected and analyzed. No manuscripts have been prepared. These tasks will be conducted upon completion of Tasks 1-4.

KEY RESEARCH ACCOMPLISHMENTS

- IRB approval obtained
- Procedures finalized for evaluating collagen fiber alignment in DCIS samples including imaging and quantification of angles
- Tumor slides have been stained for syndecan-1 expression.
- Second breast cancer diagnoses (invasive and *in situ*) have been identified among cohort participants
- Methods for statistical analysis have been established

REPORTABLE OUTCOMES

Preliminary findings regarding DCIS disease-free survival in the parent WISC Cohort were presented as a poster at the AACR Frontiers in Cancer Prevention Research annual meeting.[1]

CONCLUSION

All study procedures have been finalized. Human subjects' protection approval for this study was obtained August 2011 from the University of Wisconsin Institutional Review Board. Using H&E stained slides, tumors were imaged using second harmonic generation microscopy and fiber alignment patterns were evaluated for 16 cases. Preliminary analysis suggests that fiber alignment patterns are significantly different for DCIS lesions than for adjacent normal duct cells. Tumor slides have been stained for syndecan-1, and assessment of staining intensity has begun. Statistical data analysis of the study aims will ensue upon completion of the assessment of collagen fiber alignment patterns and syndecan-1 expression. Since data collection is not complete, no scientific knowledge or reportable outcomes have been produced yet. While we have experienced some delays with establishing procedures for evaluating fiber alignment and evaluating syndecan-1 expression, methods are in place and we expect to achieve the proposed Tasks within the project period.

REFERENCES

[1]McLaughlin V, Sprague BL, Hampton J, Newcomb P, Trentham-Dietz A. DCIS disease-free survival in the population-based Wisconsin In Situ Cohort. Presented at the Tenth AACR International Conference on Frontiers in Cancer Prevention Research, October 22-25, 2011, Boston, MA.

APPENDICES

None